Purification and Properties of an Endo-1,4-β-Glucanase from *Clostridium josui*

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An enzyme active against carboxymethyl cellulose (CMC) was purified from the stationary-phase-culture supernatant of *Clostridium josui* grown in a medium containing ball-milled cellulose. The purification in the presence of 6 M urea yielded homogeneous enzyme after an approximately 50-fold increase in specific activity and a 13% yield. The enzyme had a molecular mass of 45 kilodaltons. The optimal temperature and pH of the enzyme against CMC were 60°C and 6.8, respectively. The enzyme hydrolyzed cellotetraose, cellopentaose, and cellohexaose to cellobiose and cellotriose but did not hydrolyze cellobiose or cellotriose. A microcrystalline cellulose, Avicel, was also hydrolyzed significantly, but the extent of hydrolysis was remarkably less than that of CMC. On the basis of these results, the enzyme purified here is one of the endo-1,4-β-glucanases. The N-terminal amino acid sequence of the enzyme is Tyr-Asp-Ala-Ser-Leu-Lys-Pro-Asn-Leu-Gln-Ile-Pro-Gln-Lys-Asn-Ile-Pro-Asn-Asn-Asp-Ala-Val-Asn-Ile-Lys.

Recently, cellulolytic anaerobic bacteria have become of great interest because of possible conversion of cellulosic materials from agricultural and forestry wastes, such as rice straw, water hyacinth, and wood chips, to ethanol and other valuable compounds (9). *Ruminococcus albus*, a ruminal anaerobe having high cellulolytic activity, has been studied to specify its cellulose-solubilizing properties (3) and its cellulases (2, 4, 5). Further studies have found a novel anaerobic bacterium having crystalline cellulose-degrading activity and have designated it *Clostridium josui* (8). Its ideal culture conditions have been determined (J. Sukhumavasi, K. Ohmiya, M. Suwana-Adth, and S. Shimizu, J. Sci. Soc. Thailand, in press). These results suggested that the organism had a potent cellulose-degrading enzyme system. However, nothing is known about the properties of this cellulase system of *C. josui*, except for data in our paper stating that a cellobiose-transferring cellulase gene was cloned and expressed in *Escherichia coli* and that the translation product was purified and characterized (K. Ohmiya, T. Fujino, J. Sukhumavasi, T. Sasaki, and S. Shimizu, submitted for publication). In the present report, we describe an enzyme having activity against carboxymethyl cellulose (CMC), which was purified to homogeneity from the culture supernatant of *C. josui* in the presence of 6 M urea and further characterized.

C. josui (FERM P-9684) from compost was cultivated in a medium containing 1.5% ball-milled cellulose (BMC) at pH 6.5 and 45°C for a given period. Details of the medium composition and preparation (10) and of the cultivation conditions (Sukhumavasi et al., in press) are described elsewhere. For the enzyme assay, CMC (molecular weight, 180,000; degree of substitution, 0.6) donated from Daiichi Kogyo Seiyaku Co. (Kyoto, Japan) was used as a substrate of carboxymethyl cellulase (CMCase). The activity was determined on the basis of the viscosity change of a CMC-containing reaction mixture (5 ml of a 1% CMC solution and 1 ml of an enzyme solution) at 37°C, pH 6.8, and monitored

for 5 min by using a cone-plate type of viscometer (Tokyo Keiki Co., Tokyo, Japan), as described elsewhere (2). One unit of enzyme activity was defined as the increase of one fluidity (reverse values of viscosity: centipoise^{-1}) of a CMC solution in 1 min. For the determination of the reducing sugar in the reaction mixture, the Somogyi method (7) was employed.

The time courses for growth of the bacteria, remaining BMC, and CMCase activities in the supernatant were determined during cultivation of *C. josui* on BMC (Fig. 1). The residual amount of BMC was quantified with the anthrone-sulfuric acid reagent after depolymerization of the BMC with 60% sulfuric acid. Cell weights were determined on the basis of the turbidity at an optical density of 660 nm. Almost all of the BMC was solubilized within 30 h, and CMCase activity in the supernatant increased to the maximum at about 40 h of cultivation.

The culture supernatant (1.5 liters) having CMCase activity was dialyzed, with a cellophane membrane, against 1,000 volumes of 10 mM Tris hydrochloride buffer, pH 7.5, containing 10 mM 2-mercaptoethanol. The dialysate was loaded on a DEAE Bio-Gel A column (Bio-Rad Laboratories, Richmond, Calif.) (2.6 by 40 cm) and then equilibrated with the Tris hydrochloride buffer containing 6 M urea, which was essential to isolate the enzyme complex of interest. Linear gradient elution was conducted with the urea buffer containing NaCl (0 to 1.0 M) as the first step. The fractions (10 ml per tube) having high activity against CMC were eluted at around 0.22 M NaCl. The purity was increased 12-fold by the first step. The salt concentration in the pooled fraction was diluted 10 times with the urea buffer. The active fraction was then loaded on a Mono Q HR column (0.5 by 5 cm; Pharmacia Japan, Tokyo, Japan). The column was equilibrated with the urea buffer. A linear gradient elution with the same buffer containing NaCl (0 to 0.2 M) was conducted, and 0.5-ml fractions were collected. A symmetrical sharp single protein peak with the activity was eluted at about 0.01 M NaCl. Urea was removed from the active fraction by rechromatography on the Mono Q HR column
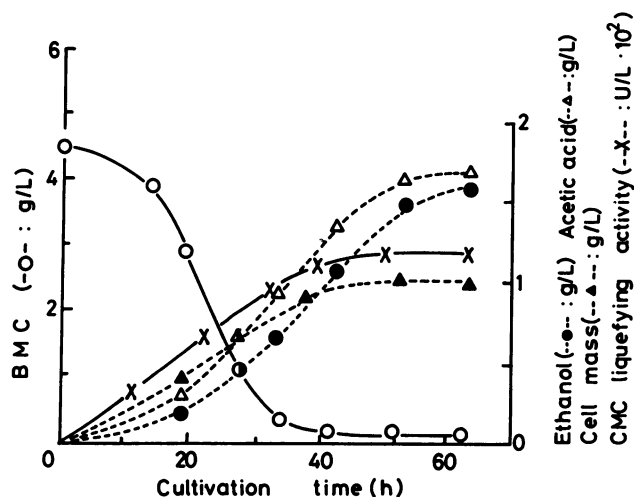
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FIG. 1. Time courses of *C. josui* BMC degradation. (○) CMC-liquefying activity, product formation (ethanol and acetic acid), and cell mass. The cultivations were carried out at pH 6.5 and 45°C in a 1-liter jar fermentor under anaerobic conditions.
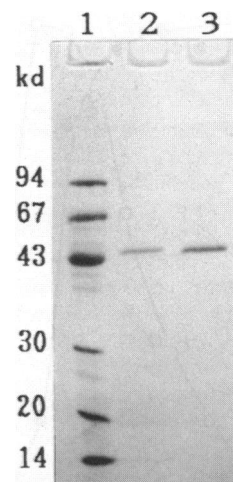

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of the enzyme. Protein was stained with Coomassie brilliant blue R-250. Lanes: 1, size marker proteins (kd, kilodaltons); 2, purified enzyme (1 μg); 3, the enzyme (3 μg).

with the urea-free buffer after purification. The final protein yield was 3 mg, with 50-fold purification and a recovery of about 13% (Table 1). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the enzyme with activity against CMC was a homogeneous protein (Fig. 2). Its molecular mass was determined to be 45 kilodaltons. Maximum activity of the enzyme was observed at pH 6.8 (Fig. 3) and at 60°C (Fig. 4). The optimal temperature (60°C) is remarkably higher than that for cultivation of *C. josui*. When the enzyme was incubated in the buffer (pH 6.8) without a substrate for 1 h, it maintained more than 80% of its initial activity at a temperature below 50°C (Fig. 4). This temperature stability was comparable to those of the CMCases of *Clostridium thermocellum* (6) and *Trichoderma reesei* (1) but was superior to the CMCase of *R. albus*. Effects of chemical reagents on the CMC-liquefying activity of the enzyme were determined (Table 2). Each reagent was added to the substrate; thereafter, enzyme was added and the reaction was allowed to proceed for 5 min at 37°C. Since both reducing reagents and sulfhydryl-reacting reagents affected the activity negligibly, sulfhydryl groups of the enzyme were not essential to reveal the activity. This property of the enzyme was significantly different from those of β-glucanases from *R. albus* (2, 4, 5).

The activity of the purified enzyme against *p*-nitrophenyl-β-D-glucoside and *p*-nitrophenyl-β-D-cellobioside was negligible when determined by the standard method described elsewhere (4, 5).

The cello-oligosaccharide-degrading reaction of the enzyme was measured by analyzing the hydrolysates of cello-oligosaccharides (Tokyo Kasei Co., Tokyo, Japan), such as

cellobiose (G2), cellotriose (G3), cellotetraose (G4), cellopentaose (G5), and cellohexaose (G6), by using high-performance liquid chromatography (BIP-1; Japan Spectroscopic Co., Tokyo, Japan) and an analytical column (Ultron NH2; Shinwa Kako Co., Kyoto, Japan). A refractive-index detector (RID-300; Japan Spectroscopic Co., Tokyo, Japan) was used for detecting cello-oligosaccharides after each saccharide (1%, 50 μl) was incubated with enzyme solution (0.002 U, 5 μl) for 12 h at 37°C (Fig. 5). The hydrolyzing activity of the enzyme against CMC and Avicel (a microcrystalline cellulose; Asahi Kasei Co., Tokyo, Japan), determined by the Somogyi method, was 1.86 and 0.07 units, respectively. The extent of this Avicel-hydrolyzing activity

TABLE 1. Summary of purification of the enzyme from *C. josui*

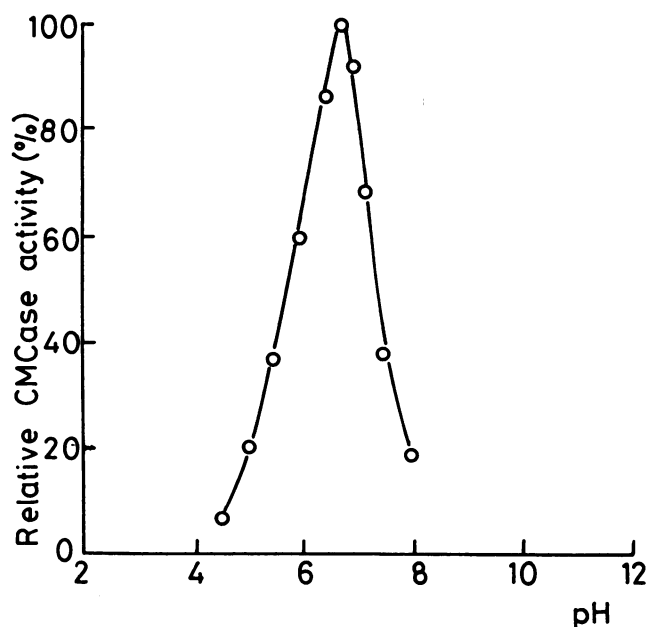| Step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Activity yield (%)	Purification (fold)
Dialysis of culture supernatant	1,097	47	0.04	100	1
DEAE Bio-Gel A	57	28	0.49	61	12
Mono Q HR	3	6	2.00	13	50

FIG. 3. Effect of pH on the CMC-liquefying activity of the purified enzyme. The pH range was as follows: acetate buffer, pH 4 to 5.5; phosphate buffer, pH 6.0 to 6.8; Tris buffer, pH 7.0 to 8.0.
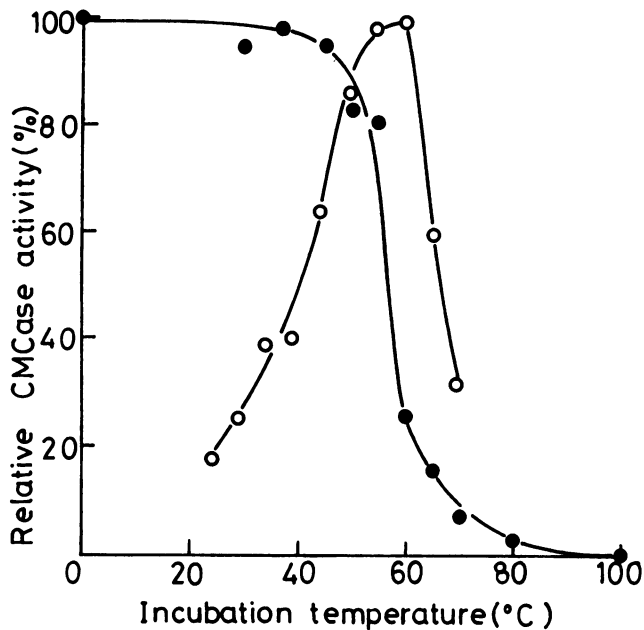
FIG. 4. Effect of temperature on the CMC-liquefying activity of the purified enzyme. Symbols: ●, thermal stability; ○, optimum temperature.

was about 1/30 of that of CMCase. In a series of experiments, G2 and G3 were negligibly degraded by the enzyme when they were analyzed by high-performance liquid chromatography. The concentration limitation for detection of compounds by this method is around 1 mM. These results are reasonable since the hydrolysates of the synthetic substrates (P-nitrophenyl-β-D-glucoside and p-nitrophenyl-β-D-cellobioside, the model compounds of G2 and G3) were also not detected, even though they are detectable at concentrations 50-fold higher than those of G2 and G3 by the colorimetric method. Judging from the chromatograms (Fig. 5), about 90% of the G4 remained, but the residual amounts of G5 and G6 were negligible, suggesting that the rate of degradation of G4 by the enzyme was very low compared

TABLE 2. Effects of chemical reagents on the CMC-liquefying activity of the enzyme

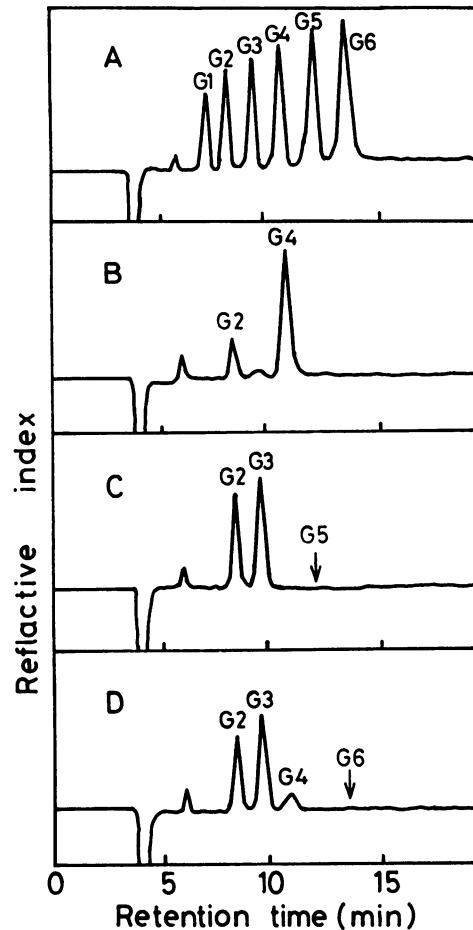| Reagent (1 mM)[a]	Relative activity (%)
None	100
Dithiothreitol	107
Cysteine hydrochloride	114
2-Mercaptoethanol	105
Glutathione	90
EDTA	110
N-Ethylmaleimide	95
Iodoacetamide	82
p-Chloromercuribenzoate	68
KCl	110
$ZnCl_2$	113
$MgCl_2 \cdot 6H_2O$	105
$CoCl_2 \cdot 6H_2O$	103
$FeCl_2$	95
$CaCl_2$	90
$CuCl_2$	69
$HgCl_2$	3

[a] Final concentration in the enzyme-substrate mixture.

FIG. 5. High-performance liquid chromatography chromatograms of the hydrolysates of cello-oligosaccharides produced by β-endoglucanase from *C. josui*. Elution was performed with a solvent system of CH_3CN-H_2O (55:45), a pressure of 40 kg/cm², and a flow rate of 0.7 ml/min at room temperature. The reaction mixtures (10 μl), incubated for 12 h at 37°C, were injected into the column (Ultron NH_2) for analysis. (A) Mixture of authentic cello-oligosaccharides of glucose (G1), cellobiose (G2), cellotriose (G3), cellotetraose (G4), cellopentaose (G5), and cellohexaose (G6). Each was at a concentration of 1%. (B) Hydrolysate of G4. (C) Hydrolysate of G5. (D) Hydrolysate of G6.

with those of G5 and G6. Therefore, the soluble substrates with higher degrees of polymerization would be better substrates for hydrolysis by the enzyme. As far as product formation was concerned, G4 was degraded mainly to G2 (Fig. 5B), and G5 was degraded mainly to G2 and G3 (Fig. 5C). Both reactions might be catalyzed by the enzyme recognizing G2. In the case of G6, the products were mainly G2, G3, and G4 but not G1, suggesting that the reactions G6 → G2 + G4 and G6 → 2 G3 might be the major ones catalyzed by the enzyme, whereas the reaction G4 → G1 + G3 might not occur. The kinetics of G4 and G5 degradation by the enzyme were also studied by using high-performance liquid chromatography. The resulting Lineweaver-Burk plots permitted calculation of K_m and V_{max} values. K_m values for the enzyme action on G4 and G5 were 15.4 and 6.7 mM, respectively. V_{max} values for the action on G4 and G5 were 1.4 and 8.1 μmol · min^{-1} · mg of protein^{-1}, respectively. The values could not be obtained for the action of the enzyme on G2 and G3, since these substrates were negligibly

hydrolyzed. Nor could they be obtained for action of the enzyme on G6, since G6 is a suspension but not a clear solution.

On the basis of the discussions in this paper and the descriptions by Wood and Bhat (11), the purified enzyme is one of the endo-1,4-β-glucanases.

The N-terminal amino acid sequence of the enzyme, was determined by using the gas-phase-amino-acid analyzer (Applied Biosystems 477A Protein Sequencer) to be as follows: Tyr-Asp-Ala-Ser-Leu-Lys-Pro-Asn-Leu-Gln-Ile-Pro-Gln-Lys-Asn-Ile-Pro-Asn-Asn-Asp-Ala-Val-Asn-Ile-Lys.

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